Amendments to the Specification

Please amend the paragraph appearing on page 2, line 16 to page 3, line 7 as follows:

--In multicellular organisms, such as humans, cells communicate with each other by the so-called signal transduction pathway, in which a secreted ligand (e.g. cytokines, growth factors or hormones) binds to its cell surface receptor(s), leading to receptor activation. The receptors are membrane proteins, which consist of an extracellular domain responsible for ligand binding, a central transmembrane region followed by a cytoplasmic domain responsible for sending the signal downstream. Signal ~~transdcution~~ transduction can take place in the following three ways: paracrine (communication between neighboring cells), autocrine (cell communication to itself) and endocrine (communication between distant cells through circulation), depending on the source of a secreted signal and the location of target cell expressing a receptor(s). One of the general mechanisms underlying receptor activation, which sets off a cascade of events beneath the cell membrane including the activation of gene expression, is that a polypeptide ligand such as a cytokine, is present in an oligomeric form, such as a homo-~~dimmer~~ dimer or trimer, which when bound to its monomeric receptor at the cell outer surface, leads to the oligomerization of the receptor. Signal transduction pathways play a key role in normal cell development and differentiation, as well as in response to external insults such as bacterial and viral infections. Abnormalities in such signal transduction pathways, in the form of either

underactivation (e.g. lack of ligand) or overactivation (e.g. too much ligand), are the underlying causes for pathological conditions and diseases such as arthritis, cancer, AIDS, and diabetes.--

Please amend the paragraph appearing on page 3, line 8 to page 4, line 10 as follows:

--One of the current strategies for treating these debilitating diseases involves the use of receptor decoys, such as soluble receptors consisting of only the extracellular ligand-binding domain, to intercept a ligand and thus overcome the overactivation of a receptor. The best example of this strategy is the creation of ~~Enbrel~~ Enbrel®, a dimeric soluble TNF-α receptor-immunoglobulin (IgG) fusion protein by Immunex (Mohler et al., 1993; Jacobs et al., 1997), which is now part of Amgen. The TNF family of cytokines is one of the major pro-inflammatory signals produced by the body in response to infection or tissue injury. However, abnormal production of these cytokines, for example, in the absence of infection or tissue injury, has been shown to be one of the underlying causes for diseases such as arthritis and psoriasis. Naturally, a TNF-α receptor is present in monomeric form on the cell surface before binding to its ligand, TNF-α, ~~which~~ which exists, in contrast, as a homotrimer (Locksley et al., 2001). Accordingly, fusing a soluble TNF-α receptor with the Fc region of immunoglobulin G1, which is capable of spontaneous dimerization via disulfide bonds (Sledziewski et al., 1992 and 1998), allowed the secretion of a dimeric soluble TNF-α receptor (Mohler et al., 1993; Jacobs et al., 1997). In comparison with the monomeric

soluble receptor, the dimeric TNF-α receptor II-Fc fusion has a greatly increased affinity to the homo-trimeric ligand. This provides a molecular basis for its clinical use in treating rheumatoid arthritis (RA), an autoimmune disease in which constitutively elevated TNF-α, a major pro-inflammatory cytokine, plays an important causal role. Although ~~Enbrel~~ Enbrel® was shown to have a Ki in the pM range (ng/mL) to TNF-α (Mohler et al., 1993), 25 mg twice a week subcutaneous injections, which translates to μg/mL level of the soluble receptor, are required for the RA patients to achieve clinical benefits (www.enbrel.com). The high level of recurrent ~~Enbrel~~ Enbrel® consumption per RA patients has created a great pressure as well as high cost for the drug supply, which limits the accessibility of the drug to millions of potential patients in this country alone.--

Please amend the paragraph appearing on page 4, line 11 to page 5, line 2 as follows:

--In addition to the TNF-α family of potent ~~proinflmmatroy~~ proinflammatory cytokines, the HIV virus that causes AIDS also uses a homo-trimeric coat protein, gp120, to gain entry into CD-4 positive T helper cells in our body (Kwong et al., 1998). One of the earliest events during HIV infection involves the binding of gp120 to its receptor CD-4, uniquely expressed on the cell surface of T helper cells (Clapham et al., 2001). Monomeric soluble CD-4 was shown over a decade ago as a potent agent against HIV infection (Clapham et al., 1989) however, the excitement was sadly dashed when its potency was shown to be limited only to laboratory HIV isolates (Daar et al., 1990). It turned out

that HIV strains from AIDS patients, unlike the laboratory isolates, had a much lower affinity to the monomeric soluble CD-4, likely due to the sequence variation on the gp120 (Daar et al., 1990). Although the dimeric soluble CD-4-Fc fusion proteins have been made, these decoy CD-4 HIV receptors showed little antiviral effect against natural occurring HIVs from AIDS patients, both in the laboratories and in clinics, due to the low affinity to the gp120 (Daar et al., 1990).--

Please amend the paragraph appearing on page 5, line 13 to page 6, line 3 as follows:

--Collagen is a family of fibrous proteins that are the major components of the extracellular matrix. It is the most abundant protein in mammals, constituting nearly 25% of the total protein in the body. Collagen plays a major structural role in the formation of bone, tendon, skin, cornea, cartilage, blood vessels, and teeth (Stryer, 1988). The fibrillar types of collagen I, II, III, IV, V, and XI are all synthesized as larger trimeric precursors, called procollagens, in which the central uninterrupted triple-helical domain consisting of hundreds of "G-X-Y" repeats (or glycine repeats) is flanked by non-collagenous domains (NC), the N- propeptide and the C-propeptide (Stryer, 1988). Both the C- and N-terminal extensions are processed proteolytically upon secretion of the procollagen ~~procallagen~~, an event that triggers the assembly of the mature protein into collagen fibrils which forms an insoluble cell matrix (Prockop et al., 1998). The shed trimeric C-propeptide of type I collagen is found in the blood of normal people at a concentration in the range of

100-600 ng/mL, with children having a higher level which is indicative with active bone formation.--

Please amend the paragraph appearing on page 7, line 21 to page 8, line 7 as follows:

--Within one embodiment, the signal peptide sequence is the native sequence of the protein to be trimerized. Within another embodiment, the signal peptide sequence is from a secreted protein different from that to be trimerized. Within one embodiment, the non-collagen polypeptide to be trimerized is a soluble receptor consisting of the ligand binding domain(s). Within one embodiment, the C-terminal portion of collagen is the C-propeptide without any triple helical region of collagen (~~Sequence IDs: 3-4~~ SEQ ID NOS:3-4). Within another embodiment, the C-terminal collagen consists of a portion of the triple helical region of collagen as linker to the non-collagenous proteins to be trimerized (~~Sequence IDs: 1-2~~ SEQ ID NOS:1-2). Within another embodiment, the C-terminal portion of collagen has a mutated or deleted BMP-1 protease recognition site (~~Sequence IDs: 3-4~~ SEQ ID NOS:3-4).--

Please amend the paragraph appearing on page 8, lines 19-23 as follows:

--In a preferred embodiment, the non-collagen polypeptide to be trimerized is the soluble TNF-RII (p75) (~~Sequence IDs: 9-12~~ SEQ ID NOS:9-12). In another preferred embodiment, the non-collagen polypeptide to be trimerized is soluble CD-4, the co-receptor of HIV (~~Sequence IDs: 13-16~~ SEQ ID NOS:13-16). In yet another preferred embodiment,

the non-collagen polypeptide to be trimerized is a placental secreted alkaline phosphatase (~~Sequence IDs: 5-8~~ SEQ ID NOS:5-8).--

Please amend the paragraph appearing on page 10, line 19 to page 11, line 9 as follows:

--The following are the advantages of this invention: (1) collagen is the most abundant protein secreted in the body of a mammal, constituting nearly 25% of the total proteins in the body; (2) the major forms of collagen naturally occur as trimeric helixes, with their globular C-propeptides being responsible for the initiating of trimerization; (3) the trimeric C-propeptide of collagen proteolytically released from the mature collagen is found naturally at sub microgram/mL level in the blood of mammals and is not known to be toxic to the body; (4) the linear triple helical region of collagen can be included as a linker with predicted 2.9 Å spacing per residue, or excluded as part of the fusion protein so the distance between a protein to be trimerized and the C-propeptide of collagen can be precisely adjusted to achieve an optimal biological activity; (5) the recognition site of BMP1 which cleaves the C-propeptide off the pro-collagen can be mutated or deleted to prevent the disruption of a trimeric fusion protein; (6) the C-propeptide ~~C-proptide~~ domain provides a universal affinity tag, which can be used for purification of any secreted fusion proteins created by this invention.--

Please amend the paragraph appearing on page 11, line 10 to page 12, line 6 as follows:

--In contrast to the Fc Tag technology (Sledziewski et al., 1992 and 1998), with which secreted dimeric fusion proteins can be created, this timely invention disclosed herein enables the creation and secretion of soluble trimeric fusion proteins for the first time. Given the fact that a homotrimer has 3-fold symmetry, whereas a homodimer ~~homodimmer~~ has only 2-fold symmetry, the two distinct structural forms theoretically can never be perfectly overlaid (Fig 1). As such, neither the homodimeric soluble TNF-R-Fc (e.g. ~~Enbrel~~ Enbrel®), nor the soluble CD4-Fc fusion proteins, could have had an optimal interface for binding to their corresponding homotrimeric ligands, TNF-α and HIV gp120, respectively. In contrast, homotrimeric soluble TNF receptors and CD4 created by the current invention are trivalent and structurally have the potential to perfectly dock to the corresponding homotrimeric ligands. Thus, these trimeric soluble receptor analogs ~~anologs~~ can be much more effective in neutralizing the biological activities of their trimeric ligands. With this timely invention, more effective yet less expensive drugs, such as trimeric soluble TNF-R and CD4 described in the preferred embodiments, can be readily and rationally designed to combat debilitating diseases such as arthritis and AIDS. Trimeric soluble gp120 can also be created with this invention, which could better mimic the native trimeric gp120 coat protein complex found on HIV viruses, and used as a more effective vaccine compared to non-trimeric gp120 antigens previously used. Also chimeric antibodies in trimeric form can be created with the current invention, which could endow greatly increased avidity of an antibody in neutralizing its antigen.--

Please amend the paragraph appearing on page 15, line 8 as follows:

--~~Sequence ID No. 1 (963 bases)~~ SEQ ID NO:1 (963 bases)--

Please amend the paragraph appearing on page 15, line 14 as follows:

--~~Sequence ID No. 2 (311 aa)~~ SEQ ID NO:2 (311 aa)--

Please amend the paragraph appearing on page 15, line 18 as follows:

--~~Sequence ID No. 3 (771 bases)~~SEQ ID NO:3 (771 bases) --

Please amend the paragraph appearing on page 16, line 1 as follows:

--~~Sequence ID No. 4 (247 aa)~~SEQ ID NO:4 (247 aa)--

Please amend the paragraph appearing on page 16, line 4 as follows:

--~~Sequence ID No. 5 (2487 bases)~~SEQ ID NO:5 (2487 bases)--

Please amend the paragraph appearing on page 16, line 9 as follows:

--~~Sequence ID No. 6 (819 aa)~~SEQ ID NO:6 (819 aa)--

Please amend the paragraph appearing on page 16, line 16 as follows:

--~~Sequence ID No. 7 (2294 bases)~~SEQ ID NO:7 (2294 bases)--

Please amend the paragraph appearing on page 16, lines 17-21 as follows:

--Nucleotide sequence encoding the human placental alkaline ~~phosphatass~~ phosphatase (AP) fused to the T2 C-propeptide human α(I) collagen (AP-T2). The bolded codons denote the start and the stop of the fusion protein. The underlined sequences indicate the restriction sites used for the fusion construct. The restriction site, which marks the fusion site shown in the middle of the sequence, is Bgl II.--

Please amend the paragraph appearing on page 16, line 22 as follows:

--~~Sequence ID No. 8 (755 aa)~~SEQ ID NO:8 (755 aa)--

Please amend the paragraph appearing on page 17, line 5 as follows:

--~~Sequence ID No. 9 (1734 bases)~~SEQ ID NO:9 (1734 bases)--

Please amend the paragraph appearing on page 17, line 11 as follows:

--~~Sequence ID No. 10 (566 aa)~~SEQ ID NO:10 (566 aa)--

Please amend the paragraph appearing on page 17, lines 12-16 as follows:

--The predicted protein sequence of the human soluble TNF-RII-T0 Fusion. The amino acid residues in blue indicate fusion sites between human soluble TNF-RII and α(I) collagen T0 polypeptide ~~polypetide~~. The underlined sequence denotes region of the "glycine repeats" upstream of the C-propeptide of human α(I) collagen. The amino acid residues in red indicate the BMP-1 protease recognition site.--

Please amend the paragraph appearing on page 17, line 17 as follows:

--~~Sequence ID No. 11 (1542 bases)~~SEQ ID NO:11 (1542 bases)-

Please amend the paragraph appearing on page 17, line 23 as follows:

--~~Sequence ID No. 12 (502 aa)~~SEQ ID NO:12 (502 aa)--

Please amend the paragraph appearing on page 18, line 5 as follows:

--~~Sequence ID No. 13 (2139 bases)~~SEQ ID NO:13 (2139 bases)--

Please amend the paragraph appearing on page 18, line 10 as follows:

--~~Sequence ID No. 14 (699 aa)~~SEQ ID NO:14 (699 aa)--

Please amend the paragraph appearing on page 18, line 16 as follows:

--~~Sequence ID No. 15 (1947 bases)~~SEQ ID NO:15 (1947 bases)--

Please amend the paragraph appearing on page 18, line 21 as follows:

--~~Sequence ID No. 16 (635 aa)~~SEQ ID NO:16 (635 aa)--

Please amend the paragraph appearing on page 21, line 5 to page 22, line 3 as follows:

--This invention discloses ways for generating such secreted trimeric receptors and biological active proteins by fusing them to the C-propeptides of collagen, which are capable of self-assembly into trimers. The following are the advantages of this invention: (1) collagen is the most abundant protein secreted in the body of a mammal, constituting nearly 25% of the total protein in the body; (2) the major forms of collagen naturally occur as trimeric helixes, with their globular C-propeptides ~~C-propetides~~ responsible for the initiating of trimerization, which are subsequently proteolytically cleaved upon triple helix formation; (3) the cleaved soluble trimeric C-propeptide of collagen is found naturally at sub microgram/mL level in the blood of mammals; (4) the linear triple helical region of collagen can be included as a linker or excluded as part of the fusion protein so the distance between a protein to be trimerized and the C-propeptide of collagen can be precisely adjusted to achieve an optimal biological

activity; (5) the recognition site of BMP1 which cleaves the C-propeptide off the pro-collagen can be mutated or deleted to prevent the disruption of a trimeric fusion protein; (6) the C-propeptides ~~C-proptide~~ domain provides a universal affinity tag, which can be used for purification of any secreted fusion proteins created by this invention; (7) unlike the IgG1 Fc tag which is known to be have other biological functions such as binding to its own cell surface receptors, the only known biological function of the C-propeptide of collagen is its ability to initiate trimerization of nascent pro-collagen chains and keep the newly made pro-collagen trimer soluble before assembly into insoluble cell matrix. These unique properties of the C-propeptide of collagen would predict that this unique trimerization tag is unlikely going to be toxic, or immunogenic, making it an ideal candidate for therapeutic applications.--

Please amend the paragraph appearing on page 22, lines 4-16 as follows:

--To demonstrate the feasibility for making secreted trimeric fusion proteins, cDNA sequences encoding the entire C-propeptides of human α1(I) containing either some glycine-repeat triple helical region (T0 construct, ~~Sequence IDs: 1-2~~ SEQ ID NOS:1-2), or no glycine-repeat with a mutated BMP-1 recognition site (T2 construct, ~~Sequence IDs: 3-4~~ SEQ ID NOS:3-4) were amplified by RT-PCR using EST clones purchased from the American Type Culture Collection (ATCC). The amplified cDNAs were each cloned as a Bgl II-XbaI fragment into the pAPtag2 mammalian expression vector (GenHunter Corporation; Leder et al.,

1996 and 1998), replacing the AP coding region (Fig. 2). The resulting vectors are called pTRIMER, versions T2 and T0, respectively. The vectors allow convenient in-frame fusion of any cDNA template encoding a soluble receptor or biologically active protein at the unique Hind III and Bgl II sites. Such fusion proteins have the collagen trimerization tags located at the C termini, similar to native pro-collagens. --

Please amend the paragraph appearing on page 22, line 17 to page 23, line 6 as follows:

--To demonstrate the feasibility of this invention, a cDNA encoding the human secreted placental alkaline phosphatase (AP), including its native signal peptide sequence, was cut out as a Hind III-Bgl II fragment from the pAPtag4 vector (GenHunter Corporation; Leder et al., 1996 and 1998) and cloned into the corresponding sites of the pTRIMER-T0 and pTRIMER-T2 vectors. The resulting AP-collagen fusion constructs (~~sequence IDs: 5-8~~ SEQ ID NOS:5-8) were expressed in HEK293T cells (GenHunter Corporation) after transfection. The successful secretion of the AP-collagen fusion proteins can be readily determined by AP activity assay using the conditioned media of the transfected cells. The AP activity reached about 1 unit/mL (or equivalent to about 1 µg/mL of the fusion protein) 2 days following the transfection. To obtain HEK293T cells stably expressing the fusion proteins, stable clones were selected following co-transfection with a puromycine-resistant vector, pBabe-Puro (GenHunter Corporation). Clones expressing AP activity were expanded and saved for long-term production of the fusion proteins.--

Please amend the paragraph appearing on page 23, line 20 to page 24, line 15 as follows:

--To provide a proof that new and therapeutically beneficial biological functions can be endowed to a trimeric fusion protein, next a trimeric human soluble TNF-RII (p75) receptor using a corresponding EST clone purchased from the ATCC was constructed. As described in Example 1, the N-terminal region of human TNF-RII, including the entire ligand-binding region, but excluding the trans-membrane domain, was cloned in-frame, as a Bam H I fragment, into the Bgl II site of both pTRIMER-T0 and pTRIMER-T2 vectors (~~Sequence IDs: 9-12~~ SEQ ID NOS:9-12). The resulting fusion constructs were expressed in HEK293T cells following transfection. Stable clones were obtained by puromycine co-selection as described in Example 1. Western blot analysis under both non-reducing and reducing conditions was carried out to determine if the resulting soluble TNF-RII-collagen fusion proteins were indeed expressed, secreted and assembled into trimeric forms. As expected, the monoclonal antibody against human TNF-RII (clone 226 from R & D Systems, Inc.) clearly recognized the trimeric soluble TNF-fusion proteins expressed by both T0 and T2 fusion vectors as 220-240 kDa bands, which are about three times bigger than the corresponding monomeric fusion proteins (Fig. 3B). The TNF-RII antibody failed to detect monomeric fusion proteins under reducing conditions, consistent with the property specified by the antibody manufacturer. As a negative control for antibody specificity, neither the HEK293T cell alone, nor the cells

expressing AP-T2 fusion protein expressed any TNF-RII (Fig. 3B).--

Please amend the paragraph appearing on page 26, line 18 to page 27, line 5 as follows:

--To create such trimeric soluble CD4 HIV receptor analogs, a cDNA encoding the entire human soluble CD4, including its native signal peptide sequence, but excluding the transmembrane and the short cytoplasmic domains, was amplified using an EST clone purchased from the ATCC. The resulting cDNA was then cloned as a Hind III-Bgl II fragment into the corresponding sites of the pTRIMER-T0 and pTRIMER-T2 expression vectors. The resulting soluble CD4-collagen fusion constructs (~~sequence IDs: 13-16~~ SEQ ID NOS:13-16) were expressed in HEK293T cells (GenHunter Corporation) after transfection. To obtain HEK293T cells stably expressing the fusion proteins, stable clones were selected following co-transfection with a puromycine-resistant vector, pBabe-Puro (GenHunter Corporation). Clones expressing the fusion proteins were expanded and saved for long-term production of the fusion proteins.--

Please amend the paragraph appearing on page 27, lines 6-15 as follows:

--To determine if the soluble ~~soublе~~ human CD4-collagen fusion proteins are assembled into disulfide bond-linked trimers, conditioned media containing soluble CD4-T0 and CD4-T2 fusions were boiled in SDS sample buffers containing either without (non-reducing) or with β-mercaptoethanol (reducing), separated by a SDS PAGE and analyzed by Western

blot using an monoclonal antibody to human CD4 (R & D Systems). Both soluble CD4-T0 and CD4-T2 fusion proteins secreted were shown to be three times as big (about 300 kDa) under the non-reducing condition as those under the reducing condition (90-100 kDa), indicating they were assembled essentially completely into homotrimers (data not shown). Now these trimeric soluble CD4 can be readily tested for gp120 binding and anti-HIV infection.--